ORIGIN OF BACTERIAL RESISTANCE TO ANTIBIOTICS[1]

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In this brief review of the problem of the genetic aspects of the origin of bacterial resistance to antibiotics, I intend to discuss mainly work done in my laboratory. I shall (1) offer evidence that bacterial resistance to penicillin and streptomycin is not induced by these compounds but originates spontaneously through genetic changes comparable to gene mutations; (2) describe the resistance patterns observed in experiments with penicillin and streptomycin; and (3) outline a possible mechanism responsible for resistance, and for the differences between the resistance patterns observed with penicillin and those observed with streptomycin.

All experiments were done *in vitro*. Penicillin was tested with *Staphylococcus aureus*, strain NRRL 313 (Demerec, 1945*a*), and streptomycin was tested with the same strain of *S. aureus* and with *Escherichia coli*, strain B. The streptomycin was obtained from Chas. Pfizer & Co., New York, at a time when this compound was still very scarce, and I wish here to acknowledge their generosity. In all the experiments bacteria were first grown in broth cultures without any penicillin or streptomycin; these were used only in the tests for resistance, made by growing bacteria on broth agar plates containing various concentrations of one or the other antibiotic.

SURVIVAL CURVES

Figures 1 and 2 show the behavior of our strain of *S. aureus* when plated on nutrient agar plates to which specified amounts of penicillin (figure 1) or streptomycin (figure 2) had been added. It is evident from these curves that on low concentrations of either antibiotic all bacteria survived and formed colonies. Threshold concentrations are indicated on the curves by sharp breaks, the numbers of survivors decreasing very rapidly with increase of concentrations. The slope of the curves is very steep at concentrations near the threshold, but levels out as concentrations become increasingly higher. These curves show that we were dealing with mixed populations of types sensitive to antibiotics and types more or less resistant. Sensitive bacteria made up by far the largest portion of the populations.

The two curves are very similar in the region of lower concentrations. A striking difference is evident, however, in the region of higher concentrations; on the medium containing streptomycin, survivors continued to appear even at the highest concentration used in the experiments.

[1] Substance of a paper delivered at the Symposium on Antibiotics at the Chicago meeting of Section N of the American Association for the Advancement of Science, December 30, 1947.

Tests of survivors confirmed the conclusion indicated by the survival curves. Strains isolated from the colonies of plates having high concentrations of either penicillin or streptomycin proved to be more resistant to the respective compound and grew on concentrations at least as high as those of the plates from which they

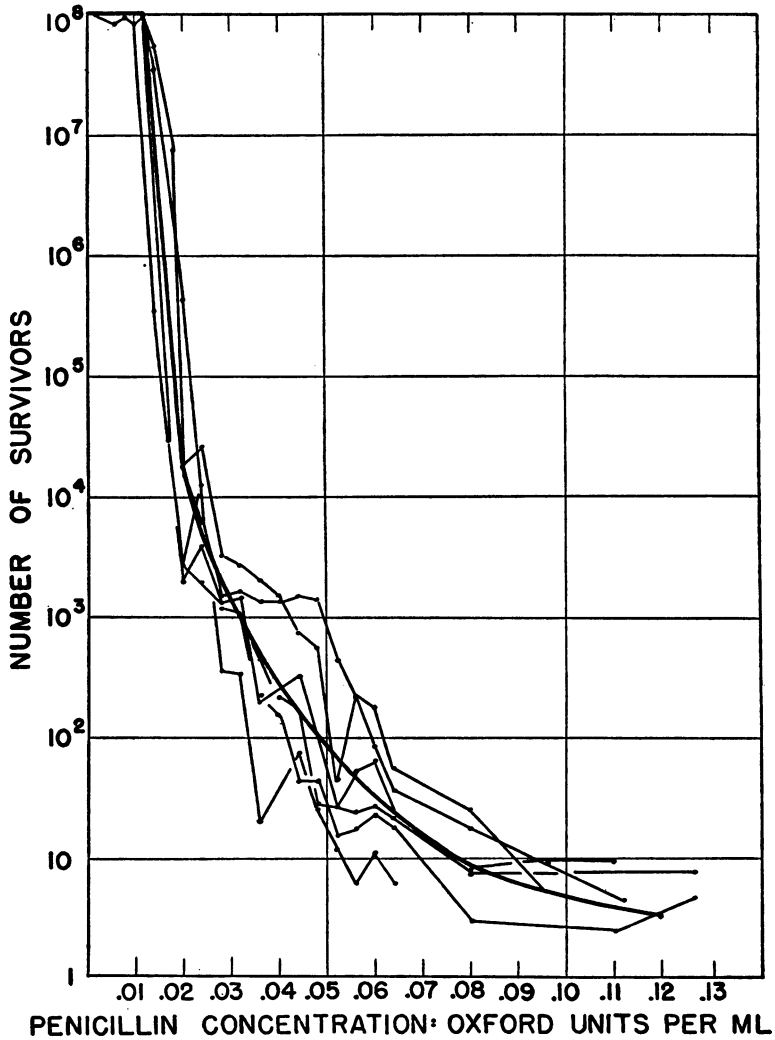

Figure 1. Survival curves for *Staphylococcus aureus* plated on nutrient agar containing various concentrations of penicillin. The six light curves represent results of six independent experiments, and the heavy curve represents the average of these experiments.

had been isolated. It was found that resistance to one of these antibiotics is independent of resistance to the other; that is to say, strains with increased resistance to penicillin are still sensitive to streptomycin, and vice versa. It seems unnecessary to discuss in detail the by now well-established fact that strains that once become resistant as a rule continue so.

ORIGIN OF RESISTANCE

The numerous experiments made in gathering the data for the survival curves showed clearly that in large populations of bacteria there were always some individuals more resistant to the antibiotics than others. Since we used very small

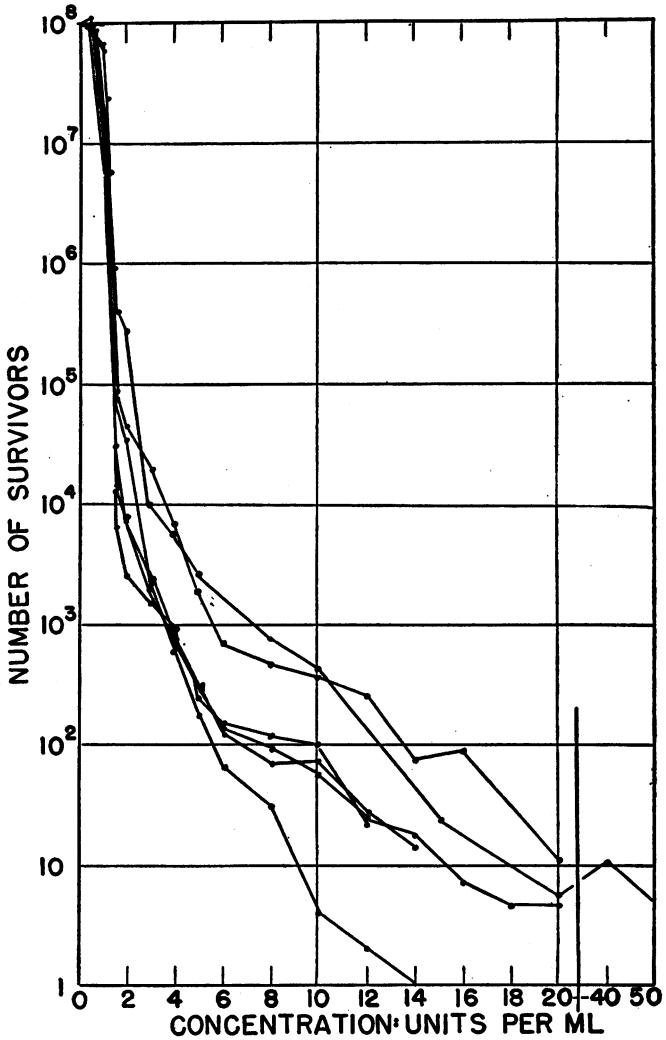

Figure 2. Survival curves for *Staphylococcus aureus* plated on nutrient agar containing various concentrations of streptomycin.

inocula (50 to 300 bacteria), the proportion of resistant bacteria was too small to account for their presence by assuming that they came about through division of one or more resistant individuals that may have been present in the inoculum. Therefore the resistant individuals must have originated in the experimental cultures. Two alternative possibilities were considered with respect to the

mechanism of this origin: (1) that resistance was induced by some interaction between the antibiotic and the bacteria when they were together on the plate; and (2) that it originated independently of the antibiotic, by mutation, the antibiotic acting only as a selective agent in the isolation of mutants by destruction of sensitive bacteria.

A relatively simple method was available for distinguishing experimentally between these two possibilities. It was devised by Luria and Delbrück (1943) in a study of the origin of bacterial resistance to bacteriophages, and was adapted for work with antibiotics in our study of the origin of resistance to penicillin (Demerec, 1945*a*). Following is a brief description of the method as used in our experiments.

From a single culture of bacteria, small inocula (50 to 300 bacteria) were taken and used to start 21 or more independent broth cultures. These were incubated for 24 hours, or until growth had reached the saturation point. During incubation the number of bacteria (in experiments using *E. coli* or *S. aureus*) increased to about 2×10^8 per ml. From the same culture that served as the source of the inocula, 10 samples of bacteria of the same size as the inocula were plated on a culture medium containing the same concentration of antibiotic as was used in the tests, in order to determine if any resistant bacteria were present in these samples. The concentration of the antibiotic had to be high enough so that there were no survivors among the small number of bacteria plated—in other words, that there were no bacteria resistant to that concentration in the inocula used to start cultures. It followed, then, that all resistant bacteria found in full-grown cultures had necessarily originated in these cultures during the period when the number of bacteria increased from 50 to 300 to about 2×10^8 per ml.

Next, from 20 of the broth cultures, samples of 0.1 ml were taken and plated on petri dishes containing the same concentration of the antibiotic (0.064 units of penicillin, or 5 units of streptomycin per ml of medium). Fifteen 1-ml samples were plated from the twenty-first tube. Thus two sets of plates were obtained: one set of 20 in which each plate had bacteria from a different culture, and another set of 15 all having bacteria from the same culture. The plates were incubated for 24 hours, or for a longer period if the growth of colonies was slow. After incubation the number of colonies on each plate was determined. These colonies represented resistant bacteria that had been present in the sample plated.

On both sets of plates in this test the experimental conditions were similar, like numbers of bacteria (about 2×10^7) having been plated onto nutrient agar containing identical concentrations of antibiotic. Therefore, if resistance were induced through interaction between the bacteria and the antibiotic when they were in contact with each other, approximately similar numbers of resistant bacteria would presumably be obtained on all the plates, regardless of the origin of the bacterial samples; the variation between plates should not exceed random variability. In the event that the origin of resistance is mutational, on the other hand, similar numbers of resistant colonies would be obtained only among the platings taken from the same culture, since these represented repeated tests of

the same mixture of resistant and sensitive bacteria. Among the samples from separate cultures, if mutations occur at random, a large number of resistant colonies would be obtained from cultures in which mutation happened to occur early in the growth of the culture, and a small number of resistant colonies from cultures in which mutation happened to occur late, provided the growth rate of resistant bacteria is not appreciably different from that of normal ones. If resistance originates by mutation, then, the variation in numbers of resistant bacteria would be much greater between samples taken from separate cultures than between samples taken from the same culture.

TABLE 1

Number of bacteria (E. coli) resistant to a concentration of 5 units of streptomycin per ml of agar medium in samples taken from a series of independent cultures and similar samples taken from a single culture which assayed 1.3 × 10^8 bacteria per ml

SAMPLES FROM INDEPENDENT CULTURES				SAMPLES FROM SINGLE CULTURE			
Culture no.	No. of resistant bacteria	Culture no.	No. of resistant bacteria	Sample no.	No. of resistant bacteria	Sample no.	No. of resistant bacteria
1	67	11	56	1	142	11	110
2	159	12	91	2	155	12	125
3	135	13	123	3	132	13	135
4	291	14	97	4	123	14	121
5	75	15	48	5	140	15	112
6	117	16	52	6	146		
7	73	17	54	7	141		
8	129	18	89	8	137		
9	86	19	111	9	128		
10	101	20	164	10	121		
Average			105.9	Average			131.2
Variance			2913.9	Variance			151.1
Chi-square			550.3	Chi-square			17.3
P much less than			0.001	P			0.26

Table 1 shows the results of such an experiment with *E. coli* and streptomycin. In addition to the tests represented in the table, the concentration of bacteria was determined in 11 cultures, including the one from which the 15 samples were taken. The average number of bacteria in 10 cultures was 2.2 × 10^8 per ml, with extreme variants of 1.9 and 2.3, and the average number in the eleventh was 2.1 × 10^8 per ml. Thus the variation in numbers of bacteria among the different cultures was so small that it could have introduced only negligible differences between the numbers of resistant colonies observed on different plates. It is evident from table 1 that the variation in number of resistant colonies was considerably greater among platings from independent cultures than among platings from a single culture. The extreme variants of independent cultures were 48 and 291, the average 106, the variance 2,914, chi-square 550, and the probability that this variation was due to chance is insignificant. On the other

hand, the variation in number of resistant colonies among platings of samples taken from one culture was very small; and the probability that this variation was due to chance is 26 per 100 trials. Very similar results were obtained in experiments using *S. aureus* and penicillin (Demerec, 1945*a*).

These results, then, favor the assumption that resistance to certain concentrations of penicillin or streptomycin originates through mutation, and that resistant bacteria may be found in any large population, the proportion depending on the mutation rate.

Oakberg and Luria (1947) reached identical conclusions after experimenting with *S. aureus* and sodium sulfathiazole. This suggests that mutations may be generally responsible for the origin of resistance that is transmitted to the offspring of the individuals that acquire it.

RESISTANCE STEPS

A very interesting feature of bacterial resistance to antibiotics is the stepwise increase in degree of resistance that can be brought about by selection. This feature is particularly well expressed in penicillin resistance. Figure 3 reproduces curves from an earlier paper (Demerec, 1945*a*) showing the effect of selection on the increase in resistance of *S. aureus* to penicillin. The first is the survival curve of the stock culture. At a concentration of 0.15 units per ml there were no survivors, but at a concentration of 0.12 units about 4 per 10^8 bacteria lived. First-step resistant strains were isolated from stock culture bacteria surviving sublethal concentrations. The second curve of figure 3 is a typical survival curve of such first-step resistant strains. Some individuals of these strains survived concentrations up to about 0.2 units. When first-step resistant strains were grown on sublethal concentrations of penicillin, second-step resistant strains were isolated from the survivors. A typical second-step survival curve is shown third on figure 3. Third- and fourth-step resistant strains were obtained in similar manner.

It is of interest to note that the building up of resistance is more rapid with each selection step. Thus, with our strain of *S. aureus*, a concentration of 0.15 units was sufficient to eliminate all bacteria of the original strain, but a concentration of about 0.2 units was required to eliminate all bacteria of the first-step resistant strain, and concentrations of about 0.4 units for the second-step, 1 unit for the third-step, and 7 units for the fourth-step. The fifth-step strain was for all practical purposes completely resistant to penicillin, since it was not affected by a concentration of 250 units per ml. With each step the increase in resistance appeared to be exponential.

Whereas the building up of resistance to penicillin followed a definite pattern, resistance to streptomycin showed a considerable degree of variability. Among first-step resistant strains—that is, among strains isolated from colonies of the original strain that survived sublethal doses of streptomycin—there were some that were only slightly more resistant than the original strain, some that were almost completely resistant, and some that fell between these two extremes (figure 4). It has been found that the variability in degree of resistance among

first-step penicillin-resistant strains (Demerec, 1945*b*) is slight as compared with the variability observed among first-step streptomycin-resistant strains.

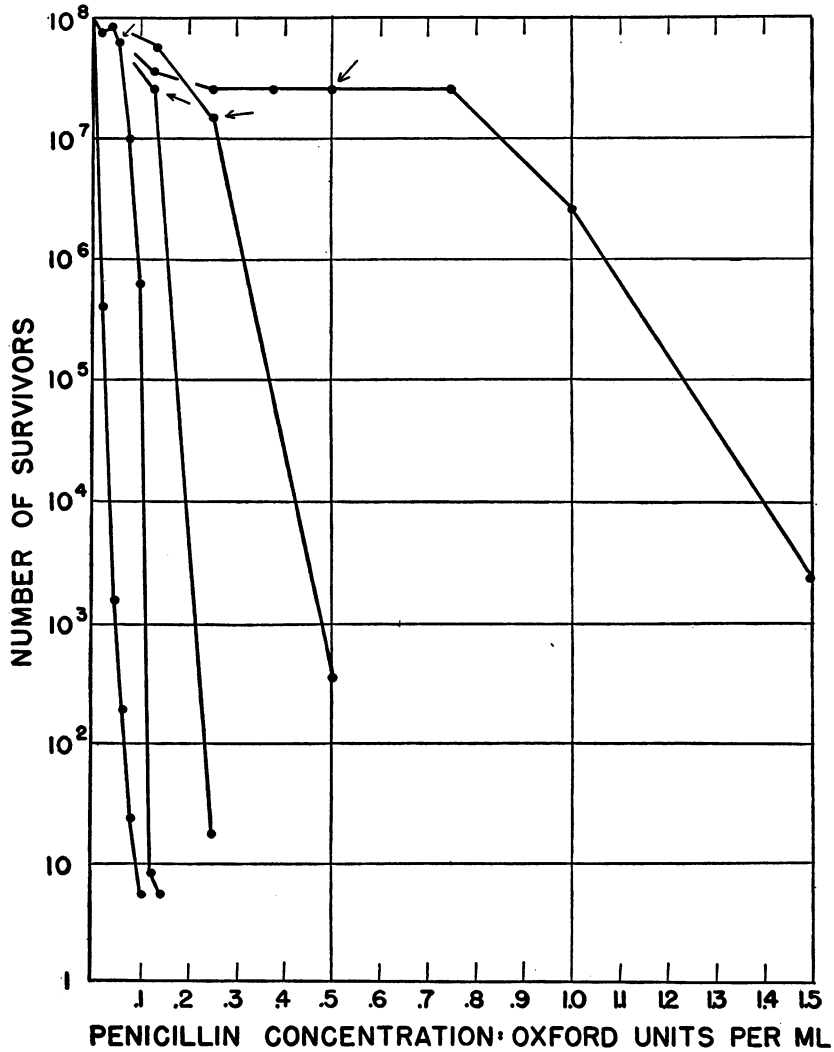

Figure 3. Stepwise build-up of resistance to penicillin in *S. aureus*. First from left, survival curve of stock culture; second, survival curve of first-step resistant strain isolated from a colony of the stock culture growing on the concentration indicated by the arrow. The other curves are of second-, third-, and fourth-step resistant strains isolated from colonies growing on the concentrations indicated by the arrows.

In the build-up of resistance to streptomycin, resistant strains of the second step, third step, etc., showed behavior similar to the first-step strains; that is, they exhibited a wide range of variability in degree of resistance. By plating bacteria of a low-resistance first-step strain on a concentration of streptomycin that is sublethal for that strain, one can isolate second-step resistant strains that

vary in degree of resistance from only slightly more resistant than the original strain to very resistant. In fact, there is no difference in degree of resistance between the most resistant strains of the first step and those of the second, third,

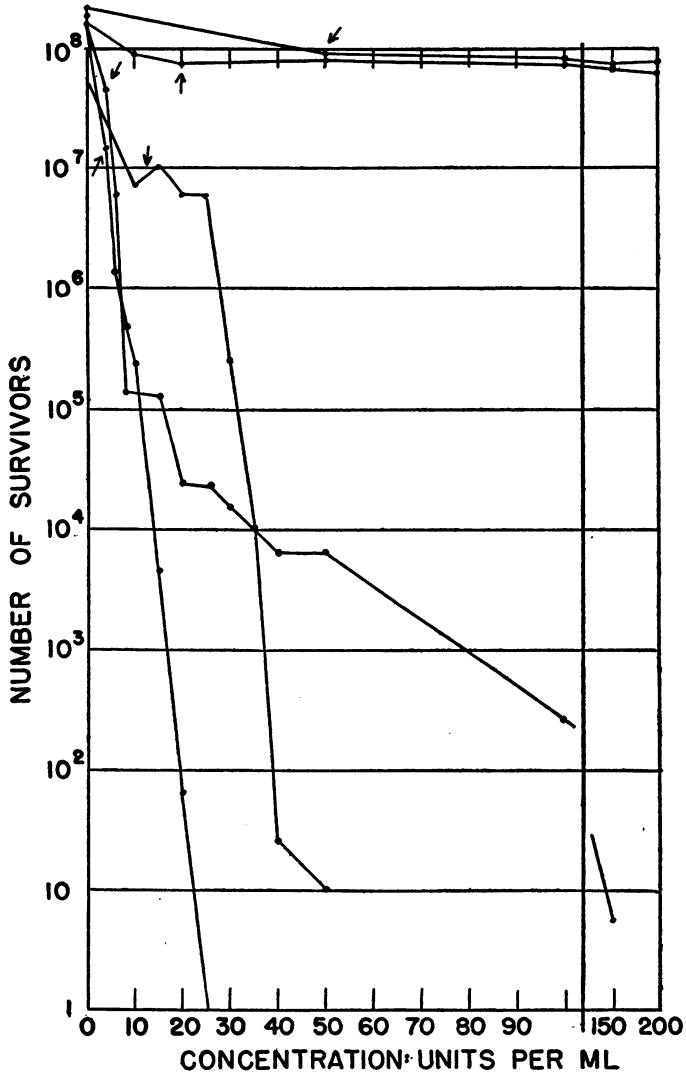

Figure 4. Survival curves of first-step strains of *S. aureus* resistant to streptomycin, showing the great variability in degree of resistance. Each strain was isolated from a colony of the stock culture growing on the concentration of streptomycin indicated by the arrow.

and higher steps. Consequently, strains that are highly resistant to streptomycin may be obtained either in one step, by selection of survivors of very high concentrations, or in several steps, by repeated selection of survivors from bacteria grown on increasingly higher concentrations.

POSSIBLE MECHANISM OF ORIGIN OF HIGH-DEGREE RESISTANCE

The experimental evidence available at present indicates that resistance to penicillin and resistance to streptomycin are independent of each other; that such resistance is a heritable property induced by genetic changes comparable to mutations; that first-step penicillin-resistant strains are fairly uniform in their degree of resistance, a highly resistant strain being built up by selection through several steps; and that first-step streptomycin-resistant strains show a great deal of variability in degree of resistance, highly resistant strains being produced either in one step by selection from among first-step resistant mutants or in several steps by repeated selection of strains having higher and higher degrees of resistance.

What mechanism is responsible for the stepwise build-up of resistance, and for the difference between penicillin resistance and streptomycin resistance in this regard? Evidence accumulated by several investigators in recent genetical research with bacteria makes it appear reasonably certain that mutations in bacteria are caused by changes in genes. Granting this assumption, the complexity of behavior observed in studies of resistance to antibiotics indicates that several genes must be involved. Such an inference is not new. Several years ago Demerec and Fano (1945) suggested that the complex situation observed in their study of resistance of *E. coli* to 7 phages pointed to the presence of about 20 distinct mutant types in their material. Since many more than 7 phages affect the strain of coli investigated, and since it is reasonable to assume that extension of the study to these phages would reveal additional mutants, it is evident that the genetic background of resistance to phages is very complex indeed, and that it involves a considerable number of genes.

If a like situation exists in respect to resistance to antibiotics, then it can be assumed that many genes are instrumental in determining resistance to the two antibiotics used in these experiments, and that the genes affecting resistance to penicillin are different from those affecting resistance to streptomycin. If any one of these genes should mutate, the bacterium in which such a mutation occurred and the strain developed from that bacterium would be more resistant to the respective antibiotic than was the original parent strain. Such a strain would be what we have called a "first-step resistant strain."

The fact that first-step penicillin-resistant strains are fairly uniform in degree of resistance (Demerec, 1945*b*, figure 2) is consistent with the assumption that all genes affecting resistance to penicillin have a similar potency, so that the effect of mutation is the same regardless of which of the genes happens to mutate. According to this hypothesis, there is still present in a first-step resistant strain a number of unmutated genes that affect resistance. Mutation of any of these produces a second-step resistant strain, which possesses a higher degree of resistance than the first-step strain. Similarly, by mutation of another gene in a second-step resistant strain, a still higher degree of resistance is attained, characteristic of the third-step resistant strain. From this, by further mutations, highly resistant fourth- and fifth-step strains may be obtained. The curves in figure 3 indicate that the increase in degree of resistance with each step is ex-

ponential. This means that the effect of two or more mutants together is considerably greater than would be expected from the added values of the effects of single mutants.

No attempt has been made to determine the frequency with which genes affecting resistance to penicillin mutate. It may be estimated from survival curves, however, that the mutation rate is low, in the neighborhood of 1×10^{-8}. With such a low mutation rate, it is evident that the increase in resistance must occur in successive steps, and that the chance that one step will be skipped is very slight, the chance that two steps will be skipped practically nil. (A step would be skipped when mutations in two genes occurred simultaneously in the same bacterium; and two steps would be skipped if three mutations occurred simultaneously in a single cell. The chance of two simultaneous mutations is $10^{-8} \times 10^{-8} = 10^{-16}$, and of three simultaneous mutations, $10^{-8} \times 10^{-8} \times 10^{-8} = 10^{-24}$. Since the volume of an *S. aureus* cell is about one cubic micron, it would be expected that one double mutant, on the average, would be found in ten liters of bacteria, and one triple mutant in one million cubic meters.)

The observed behavior of resistance to streptomycin also can be explained by assuming the existence of several genes determining such resistance. Unlike the genes for penicillin resistance, however, these differ greatly from one another in potency. If a gene of low potency mutates, the first-step resistant strain will have a low degree of resistance, but if mutation occurs in a highly potent gene, the first-step resistant strain will be highly resistant. Consequently, considerable variation in degree of resistance is to be expected between first-step strains; and for the same reason a highly resistant strain may be obtained either in one step, by selection of a highly resistant first-step mutant, or in several steps, by selection of mutants of low resistance values.

CLINICAL CONSIDERATIONS

A major consideration in the clinical use of antibiotics is how to avoid the development of resistant strains, since the usefulness of an antibiotic is closely related to the number of resistant pathogens and to their incidence in infections. For this reason, analysis of the mechanism of origin of resistance to penicillin and streptomycin has an important bearing on the clinical application of these antibiotics.

From the clinical standpoint, the situation in regard to penicillin is relatively simple and well defined. Since resistance develops in steps, and it is very unlikely that a step will be skipped in the process, the clinician can avoid development of resistant pathogens by using initial doses that are adequate for the elimination of first-step resistant individuals. Fortunately, most of the common pathogenic strains that have been investigated (North and Christie, 1945; Meads *et al.*, 1945) are very sensitive to penicillin, so that large doses are not required in clinical use. It is equally important for the clinician to maintain the effective concentration in treatment as long as the infection persists, because decrease of the concentration below the effective level will permit the accumulation of first-step resistant bacteria, which may increase to a point that will allow the occurrence of

second-step mutants. These are difficult to control. If there is any suspicion that a pathogen may be more resistant to penicillin than the usual strains, it is advisable to determine the degree of resistance before starting treatment, and to adjust the concentration accordingly. If adequate precautions are taken against the development of second-step resistant bacteria, there should be no danger from resistant pathogens in penicillin treatment. It is particularly important to avoid the indiscriminate use of penicillin, however, especially for applications where it can scarcely be of any help (for example, as a mouth wash), because there is positive danger that such use may stimulate the development of resistant strains.

In the clinical use of streptomycin, the situation can be controlled to a much smaller extent. Since highly resistant bacteria are found among the first-step mutants, treatment with high concentrations is not effective in eliminating the whole population of bacteria present in an infection. What it does accomplish is a reduction of the number of bacteria to a level with which the organism is capable of dealing. If for some reason the organism cannot do this, the chances for the development of a resistant strain are exceedingly good. Therefore, it must be expected that pathogenic strains resistant to streptomycin will frequently develop, in the course of time replacing sensitive strains in communities where streptomycin is used and rendering this antibiotic ineffective. This eventuality can be postponed by restricting the use of streptomycin to serious infections which cannot be controlled in any other way.

It may be appropriate to mention here the most effective way, theoretically, of preventing the origin of resistant strains of bacteria. This is the use in clinical treatment of a mixture of two antibiotics, when such are available, that affect the same pathogen but are independent in their actions. The evidence of independence is that bacterial strains that have developed resistance to one antibiotic are still sensitive to the other, and vice versa. If such a mixture of two antibiotics is used, then only bacteria that are resistant to both can survive the treatment and form first-step resistant strains. Such bacteria would be exceedingly rare. For example, if first-step resistant bacteria for each of two antibiotics should be found in a large population with a frequency of 1×10^{-7}, then the expected frequency of bacteria resistant to both these antibiotics would be 1×10^{-14}.

SUMMARY

A method is described that has been used to determine whether resistance to streptomycin is induced by interaction of the compound with bacteria or originates by gene mutation. Data are presented indicating that mutations are responsible for the origin of streptomycin resistance in *Staphylococcus aureus*. These agree with previously published data regarding the origin of penicillin resistance in the same organism.

The stepwise increase of resistance to penicillin by selection is explained by assuming that mutations in several equally potent genes are effective in inducing resistance, and that the slight degree of resistance characteristic of the first step is due to a mutation in one of these genes, the higher degrees of resistance of subsequent steps to successive mutations in other genes.

The increase in resistance to streptomycin also can be explained by the assumption that several genes are instrumental in the process. These genes vary greatly in their potency, however, and consequently a mutation in a highly potent gene will be responsible for a high degree of resistance, a mutation in a less potent gene for a low degree of resistance.

From the knowledge gained concerning the mechanism of origin of resistance, it is concluded that in treatment with penicillin the development of highly resistant strains can be avoided by application of the penicillin in doses sufficiently large to prevent survival of first-step resistant mutants. In treatment with streptomycin, however, the development of highly resistant strains cannot be prevented; effective treatment does not eliminate all bacteria, but it probably reduces their number to a level at which the organism is able to eliminate them.

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